Biochimica et Biophysica Acta 1808 (2011) 1601–1610

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Hetero-oligomeric cell wall channels (porins) of *Nocardia farcinica*

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ARTICLE INFO

Article history:
Received 9 September 2010
Received in revised form 5 November 2010
Accepted 8 November 2010
Available online 16 November 2010

Keywords:
Cell wall channel
Porin
MspA
Mycolic acid
Lipid bilayer membrane
N. farcinica

ABSTRACT

The cell wall of *Nocardia farcinica* contains a cation-selective cell wall channel, which may be responsible for the limited permeability of the cell wall of *N. farcinica* for negatively charged antibiotics. Based on partial sequencing of the protein responsible for channel formation derived from *N. farcinica* ATTC 3318 we were able to identify the corresponding genes (*nfa15890* and *nfa15900*) within the known genome of *N. farcinica* IFM 10152. The corresponding genes of *N. farcinica* ATTC 3318 were separately expressed in the *Escherichia coli* BL21DE3Omp8 strain and the N-terminal His_{10}-tagged proteins were purified to homogeneity using immobilized metal affinity chromatography. The pure proteins were designated $NfpA_{NHis}$ and $NfpB_{NHis}$, for *N. farcinica porin A and N. farcinica porin B.* The two proteins were checked separately for channel formation in lipid bilayers. Our results clearly indicate that the proteins $NfpA_{NHis}$ and $NfpB_{NHis}$ expressed in *E. coli* could only together form a channel in lipid bilayer membranes. This means that the cell wall channel of *N. farcinica* is formed by a heterooligomer. NfpA and NfpB form together a channel that may structurally be related to MspA of *Mycobacterium smegmatis* based on amino acid comparison and renaturation procedure.

1. Introduction

The gram-positive bacterium *Nocardia farcinica*, together with *Nocardia nova* and *Nocardia asteroides sensu stricto*, belongs to the so-called *N. asteroides* complex whose members cause the majority of human nocardioses [1].

Nocardia is a genus of gram-positive, strictly aerobic rod-shaped bacteria belonging to the family Nocardiaceae of the suborder Corynebacterineae and is mainly found in soil and water and even in animal tissues [2]. At present many isolates are known, which can be subdivided in non-pathogenic and pathogenic strains. Pathogenic *Nocardia* strains can cause pulmonary nocardiosis, systemic nocardiosis and local extrapulmonary infections implying various clinical symptoms [1,4,5]. Recently, *N. farcinica* was the most frequently isolated species of Nocardiae in Japan and represented the main causative organism for nocardiosis (http://nocardia.nih.go.jp/) [3]. In many cases, predisposing factors such as underlying immunocompromising conditions including AIDS or immunosuppressive treatment may facilitate infection although nocardiosis may also occur in immunocompetent patients [6]. The intrinsic, multiple drug resistance is a serious problem for the treatment of nocardiosis because it heavily relies on chemotherapy. On the other hand, there exists also some interest in certain *Nocardia* species because some of them are known to produce antibiotics and others degrade or convert aromatic compounds [7,8].

N. farcinica and other nocardiae belong to the mycolic acid containing actinomycetes, also known as mycolata [9]. The mycolata comprise microorganisms, which cause the most dangerous infections worldwide such as *Mycobacterium tuberculosis* (TBC, at present 3 million deaths/a), *Mycobacterium leprae* (lepra), *Nocardia farcinica* (nocardiosis) and *Corynebacterium diphtheriae* (diphtheria) as well as microorganisms used for the production of amino acids on industrial scale such as *Corynebacterium glutamicum* and *Corynebacterium efficiens* (glutamate production; worldwide about 340.000 t/a). Due to their heterogeneity, their extreme acid resistance and their unusual cell wall structure of the mycolata represent an interesting and challenging research object because the cell wall represents an additional permeability barrier for hydrophilic solutes besides the cytoplasmic membrane [10,11].

The permeability barrier of the cell wall is formed by long mycolic acids covalently linked to the arabinogalactan and additional free lipid associated with the mycolic acid layer [12]. Consequently, the cell wall of the mycolata resembles the function of the outer membrane of gram-negative bacteria forming an efficient permeability barrier composed of lipids and lipopolysaccharides [13].

Hence, channels for the passage of hydrophilic compounds have been identified in the cell wall of some members of the mycolata such as *Mycobacterium chelonea* [14], *C. glutamicum* [15] and *Nocardia*

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doi:10.1016/j.bbamem.2010.11.011

farcinica [16]. These channels are wide, water-filled and contain negative point charges. Cell wall channels are involved in drug uptake, because the three important antimycobacterial drugs isoniazide, ethambutol, and pyrazinamide can easily permeate through the MspA channel of *Mycobacterium smegmatis* [17]. Eight MspA molecules form the best studied outer membrane channel of the mycolata [18]. So far, there is no significant homology between MspA and other known polypeptide subunits forming cell wall channels from other species from mycolata, such as *C. glutamicum* [19] or *C. diphtheriae* [20].

In this study, we were able to identify six subunits of the cell wall channel of *N. farcinica*, which all code for proteins of about 20 kDa. Two subunits, $NfpA_{NHis}$ and $NfpB_{NHis}$, of the cell wall channel were heterologous expressed in *Escherichia coli* and purified to homogeneity. The two subunits form only *together* but not alone a cell wall channel that may be structurally related to the channel formed by MspA [18].

2. Materials and methods

2.1. Bacterial strains and growth conditions

N. farcinica N 236 IMMIB (ATCC 3318) was used in all the experiments. The strain was grown in 500 ml Erlenmayer flasks containing 250 ml Brain Heart Infusion (BHI) broth at 36 1 °C using a New Brunswick shaker at 120 rpm for 7 days. Cells were killed by adding 1% formaldehyde to the growth medium and harvested by centrifugation at 4000 rpm for 10 min.

E. coli strains were grown in LB medium or on LB agar plates at 37 °C with appropriate antibiotics (Sigma). *E. coli* strains Top10F′ (Invitrogen) and Neb5α (New England Biolabs) were both used for the cloning procedure, whereas BL21DE3Omp8 [21] was utilized for the expression experiments. 100 μg/ml ampicillin, 40 μg/ml kanamycin and 25 μg/ml chloramphenicol were used for selection.

2.2. Isolation of the cell wall channel protein

The cell wall channel of *N. farcinica* N 236 IMMIB (ATCC 3318) was isolated and purified essentially as has been described previously [16]. In brief, the cell pellet was washed with physiological saline and centrifuged again. The cells were then resuspended in sterile distilled water and homogenized using glass beads with a diameter of 0.17–0.18 mm. The cell walls were prepared as described previously [22]. The cell wall pellet was treated with a number of different buffers to remove most of the soluble cell wall components. Final purification of the channel-forming protein was achieved by excision of this band from tricine-containing preparative SDS-PAGE and its extraction with 0.4% LDAO, 10 mM Tris–HCl, pH 8. Using this method the protein appeared to pure.

2.3. Peptide sequencing

The purified protein with an apparent molecular mass of about 87 kDa was precipitated using trichloroacetic acid to remove the detergent. The amino acid sequence of the peptide was determined by the Edman degradation method using a gas phase sequenator (470A, Applied Biosystems) with on-line detection of the amino acids. Cyanobromide (CNBr) cleavage of the 87 kDa protein was performed according to [23]. The cleaved peptides were purified by FPLC followed by Edman degradation.

2.4. Cloning of the genes nfpA, and nfpB in porin deficient BL21DE3Omp8 E. coli strain

Chromosomal DNA was used from *N. farcinica* N 236 IMMIB (ATCC 3318) in all the experiments. The expression plasmid pARAJS2 is a derivative of pET12a (Novagen) [24] and was used for genetic complementation and expression experiments. Besides the ampicillin resistance the vector contained the multi cloning site (MCS) with the relevant restriction sites *Nco*I and *Bsp1407*I in 5′ to 3′ orientation. The genes *nfpA* (i), and *nfpB* (ii), were amplified with standard PCR methods in 25 μl reactions with 1× Taq buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase and 0.4 μM primers (see Table 3: (i) FP *nfpA Nco*I and RP *nfpA Bsp1407*I; *Bsp1407*I; (ii) FP *nfpB Nco*I, RP *nfpB* Bsp*1407*I). The PCR conditions were: initial denaturing at 95 °C for 10 min, 30 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min and final extension at 72 °C for 10 min. PCR of FP *nfpA Nco*I and RP *nfpA Bsp1407*I led to a specific 649 bp PCR of FP *nfpB Nco*I 1st, and RP *nfpB Bsp1407*I to a 642 bp fragment.

The first amplified fragment (649 bp) represents *nfpA* including 48 base pairs encoding the very first 16 amino acids upstream of the putative cleavage site (see Fig. 7) for the signal peptidase. In contrast, the 642 bp fragment represents *nfpB* (*nfa15900*) including twelve base pairs encoding the very first four amino acids upstream of the putative cleavage site for the signal peptidase. According to a successful expression of MspA monomer without putative signal peptide in porin deficient BL21DE3Omp8 *E. coli* strain [25], gene *nfpA* (*nfa15890*) was also cloned without putative signal peptide before inserting in pARAJS2. However, no difference of channel-forming properties could be observed between both $NfpA_{NHis}$ (Nfa15890) variants.

The amplified fragments *nfpA*, and *nfpB* were cloned either into the vector pJet (Fermentas) or pCR®2.1-TOPO® (Invitrogen), resulting in the plasmids pCR®2.1-TOPO®_*nfpA* and pJET1_*nfpB.* The ligation products were transformed into *E. coli* TOF10F′ and *E. coli* Neb5α cells via heat-shock and the correct plasmid recombinants were determined by colony PCR using the primer pair FP pJet1/RP pJet1 and FP M13/RP M13, respectively. All plasmids were checked by sequencing (SEQLAB, Göttingen). Furthermore, pCR®2.1-TOPO®_*nfpA* and pJET1_*nfpB* were digested with *Nco*I and *Bsp1407*I (at a ratio of 1:1, 1*Tango buffer), and ligated into *Nco*I and *Bsp1407*I treated pARAJS2 vector. The ligation procedure led to the expression vectors pARAJS2_*nfpA* and pARAJS2_*nfpB,* containing the recombinant genes $nfpAN_{His}$ and $nfpB_{NHis}$, respectively. All plasmids were sequenced prior to the transfection of BL21DE3Omp8 *E. coli* cells.

Competent *E. coli* BL21DE3Omp8 cells were transformed with the plasmids pARAJS2_*nfpA* and pARAJS2_*nfpB* according to a slightly modified standard electro-transformation method via electroporation at 600 Ω, 25 μF und 2.5 kV in a multiporator (Eppendorf).

2.5. Purification and expression of the recombinant proteins $NfpA_{NHis}$ and $NfpB_{NHis}$

The porin deficient BL21DE3Omp8 *E. coli* cells containing one of the expression plasmids were grown at 37 °C in LB medium to an OD_{600} of 0.5–0.7 using the appropriate selection of antibiotics. Then the cells were transferred to room temperature (RT) and expression was induced after 30 min of adaptation by adding 0.02% arabinose to the culture media. After 16 h cells were collected by centrifugation at 5000×*g* for 10 min at 4 °C and resuspended in 10 mM Tris pH 8. Protease inhibitor cocktail (Calbiochem) was added before disrupting the cells by a French pressure cell. Cell debris was collected by centrifugation at 5000×*g* for 10 min at 4 °C. The supernatant was ultra-centrifuged at 48,000×*g* for 1 h at 4 °C to obtain the membrane pellet. Nfp monomers were purified from the supernatant as well as from the membrane pellet. For purification under native conditions the membrane pellet was resuspended in 10 mM Tris, 2% Triton pH 8 and protease inhibitor cocktail set II (Calbiochem) was added before shaking the suspension for 30 min at RT followed by ultra-centrifugation at 48,000×*g* for 30 min at 4 °C. Isolation from the supernatant under native conditions was performed suspending 1 ml of the above described membrane supernatant in 4 ml 10 mM Tris/200 μl Ni-